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BIOSYNTHESIS AND FUNCTION OF LFA-3 IN HUMAN MUTANT CELLS DEFICIENT IN PHOSPHATIDYLINOSITOL-ANCHORED PROTEINS[1]

NURIT HOLLANDER,[2] PERIASAMY SELVARAJ, AND TIMOTHY A. SPRINGER

From the Laboratory of Membrane Immunochemistry, Dana Farber Cancer Institute and Harvard Medical School, Boston, MA 02115

Mutants that lack expression of phosphatidylinositol (PI)-anchored proteins were derived from the human B lymphoblastoid JY cell line. It was demonstrated that unlike wild-type cells, which normally express both a transmembrane and a PI-linked form of LFA-3 glycoprotein, the mutant cells expressed only the transmembrane form of LFA-3. [^{3}H]Ethanolamine was not incorporated into LFA-3 of mutant cells, indicating that the anchor moiety was entirely missing. Blockade of normal biosynthesis of the PI-anchored form led to accumulation of two intermediates that may have intact and truncated polypeptide chains. The truncated LFA-3, which was not attached to the cell membrane, was secreted by mutant cells into culture supernatants.

A possible division of adhesion function between the two forms of LFA-3 was studied by using the JY cell lines as targets for CTL. Wild-type and mutant JY cells formed conjugates with CTL and were subsequently lysed to a similar extent. In addition, wild-type and mutant JY cells stimulated CTL proliferation to the same extent. Antibody-blocking experiments demonstrated a predominant role for the CD2/LFA-3 pathway in interaction of both wild-type and mutant cells with CTL. Because E exclusively express only the PI-linked LFA-3 form, and this form is known to mediate cell adhesion, the present results indicate that the two distinct membrane-anchored LFA-3 forms are each capable of mediating adhesion. A possible division of signaling functions between the two forms of LFA-3 is under investigation.

Cell surface glycoproteins are commonly anchored in the membrane via a hydrophobic peptide domain, which penetrates the lipid bilayer, and a hydrophilic cytoplasmic domain. However, certain cell-surface proteins lack a transmembrane polypeptide domain and are anchored to the cell membrane via a C-terminally attached glycosyl-PI[3] moiety (1). PI-anchored proteins can be released from the membrane by PIPLC (1). Their predicted sequence from cDNA includes a hydrophobic C-terminal peptide domain that is absent from the fully processed mature molecules (2). The extra C-terminal protein sequences are removed soon after translation (2, 3) and are believed to function as signals for attachment of the preformed PI anchor (2).

The physiologic significance of the novel PI anchor is yet unknown. PI-attached proteins are functionally diverse. However, some of them like NCAM and LFA-3 serve as ligands in cell-cell interactions (4–9) whereas some of them like Thy-1 and Ly-6/T-cell activating protein may be involved in signal transduction and cell activation (10–12). LFA-3, an adhesion glycoprotein important in immunoregulation, is expressed on the cell surface of nucleated cells in both a polypeptide chain-anchored form and a PI-anchored form (13–15). In addition to mediating intercellular adhesion by binding to its ligand CD2 (6–9), LFA-3 may also be involved in signal transduction (16). However, the functional relationship between the two distinct membrane-anchored LFA-3 forms is unclear. To study this issue, we selected mutant human cells, which are deficient in PI-linked cell surface proteins and thus express the polypeptide-anchored form but not the PI-anchored form of LFA-3. These cell lines are models for the study of PNH, an acquired defect in hemopoietic stem cells, selectively affecting PI-anchored proteins. LFA-3 biosynthesis and function in these mutant cell lines is described in the present paper.

MATERIALS AND METHODS

Cells. The human B lymphoblastoid cell line JY and the mutant lines derived from it were maintained in RPMI 1640 (GIBCO, Grand Island, NY) medium supplemented with 10% FCS (GIBCO), 5×10^{-5} M 2-ME, and 50 μg/ml gentamicin. The generation and maintenance of the DPw2-specific CTL clone 8.2 were previously described (17). In brief, cryopreserved cells were thawed and then stimulated with 2000 rad-irradiated DPw2$^+$ PBML in medium consisting of RPMI 1640 supplemented with 10% human plasma and 15% PHA-induced T cell supernatant (17). Cells were used for cytotoxicity and conjugate assays after 5 to 6 days of incubation. PBML were isolated from human blood by flotation on Ficoll-Hypaque.

MAb. The following mouse anti-human mAb were used: TS2/9 specific for LFA-3 (18), 1A10 specific for DAF (19), 17D6 specific for BLAST 1 (20), TS2/18 specific for CD2 (18), TS1/18 specific for the β-subunit of LFA-1 (18), TS1/22 specific for the α-subunit of LFA-1 (18), and W6/32 specific for HLA class I (21). X63, a myeloma IgG, was used as a nonbinding control.

Immunofluorescence flow cytometry. Cells were analyzed for surface Ag as previously described (22).

Selection of DAF-negative variants. JY cells were cultured in 150 μg/ml ethylmethanesulfonate (Sigma Chemical Co., St. Louis, MO) for 24 h, then washed and cultured for 10 additional days. A total of 2×10^7 cells were fluorescein-labeled with DAF mAb. Cells expressing no detectable DAF were then separated by sorting for 0.5% of the most negative cells, using an Epics V flow cytometer

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[1] This work was supported by the Dorot Foundation, an American Cancer Society Faculty Award, and National Institutes of Health Grant CA-31798.
[2] On leave from the Department of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel.
[3] Abbreviations used in this paper: PI, phosphatidylinositol; DAF, decay-accelerating factor; PBML, peripheral blood mononuclear leukocytes; PIPLC, phosphatidylinositol-specific phospholipase C; PNH, paroxysmal nocturnal hemoglobinuria.

(Coulter Electronics Inc., Hialeah, FL). Sorted cells were allowed to expand in culture, and the selection process was repeated two more times, until most of the selected cells were negative for DAF expression. At this stage cells were cloned by limiting dilution. PBML treated with mitomycin C (40 μg/ml for 1 h at 37°C and three washes) were used as feeder cells for cloning (2×10^5/well). Resulting clones were analyzed for DAF expression by immunofluorescence flow cytometry.

PIPLC treatment of cells. PIPLC purified from *Staphylococcus aureus* (23) was used. JY cells were incubated at 37°C for 1 h in Dulbecco's modified PBS containing 1 mg/ml OVA and 10 μg/ml of PIPLC. As a control, cells were incubated under identical conditions but without PIPLC. Cells were then washed and analyzed for surface Ag by immunofluorescence flow cytometry. ^{125}I surface labeled cells were similarly subjected to PIPLC treatment. Culture supernatants were then centrifuged at 100,000 × *g* for 1 h and analyzed for protein release by immunoprecipitation.

Radioisotopic cell labeling. Cells were surface labeled with ^{125}I by using 1,3,4,6-tetrachloro-3,6-diphenylglycoluril (Pierce Chemical Co. Rockford, IL) (24). For biosynthetic labeling with methionine, cells were washed and incubated at 2×10^6/ml for 1 h in methionine-free RPMI 1640 supplemented with 10% dialyzed FCS. [^{35}S]Methionine (100 μCi/ml) (New England Nuclear, Boston, MA) was then added and cells were cultured for 6 h. In pulse-chase experiments cells were pulsed for 15 min and then either collected by centrifugation or chased by addition of 1 mM methionine and incubation for an additional hour. For biosynthetic labeling with ethanolamine or with palmitic acid 2×10^6 cells/ml were incubated in RPMI 1640 plus 5% dialyzed FCS with [^{3}H]palmitic acid (250 μCi/ml) (New England Nuclear) for 16 h or with [^{3}H]ethanolamine (20 μCi/ml) (Amersham, Arlington Heights, IL) for 24 h. Labeled cells were washed three times and lysed at 2×10^7/ml with 1% Triton X-100 in 25 mM Tris, HCl/0.15 M NaCl/0.02% NaN3, pH 8.0, containing 1% bovine hemoglobin, 1 mM PMSF, 5 mM iodoacetamide, and 1% aprotinin. Lysates were centrifuged at 12,000 × *g* for 15 min to remove insoluble material. Culture media were centrifuged at 100,000 × *g* to remove cell membranes. Supernatants were then used for immunoprecipitation.

Immunoprecipitation and gel electrophoresis. LFA-3 and HLA were immunoprecipitated from precleared samples by addition of 30 μl of a 1:1 slurry of TS2/9-Sepharose or W6/32-Sepharose (8) and incubation for 4 h at 4°C with constant shaking. Sepharose beads were then washed as described (13) and immunoprecipitated proteins were eluted from beads by boiling in 0.5% SDS for 10 min. The eluted immunoprecipitates were diluted 10-fold with 1% Triton X-100 and immunoprecipitated with a second aliquot of mAb-Sepharose. The second cycle of immunoprecipitation was to reduce background bands (25). Sample buffer was then added and samples were subjected to SDS 10% PAGE (26) under reducing conditions. The gels were dried and autoradiographed (for ^{125}I-labeled proteins) or were soaked in 2,5-diphenyloxazolyl-dimethyl sulfoxide (27) and fluorographed (for ^{35}S- and ^{3}H-labeled proteins).

N-glycanase treatment. LFA-3 samples eluted from MAb-Sepharose were incubated for 20 h with *N*-glycanase (peptide; *N*-glycosidase F) according to the supplier's instructions (Genzyme, Boston, MA). The proteins were precipitated with acetone in the presence of 10 μg of tRNA as carrier and were resuspended in sample buffer before SDS-PAGE.

Cell-mediated lysis. Target cells were incubated with 100 μCi of $Na^{51}CrO_4$ for 1 h and washed three times. They were then plated in triplicate at 5×10^3 cells/well with 2.5×10^4 effector cells/well in V-well microtiter plates in a total volume of 0.2 ml/well. The plates were incubated at 37°C for 4 h. After incubation, the plates were centrifuged at 400 × *g*, and 0.1 ml of the sample supernatants was harvested and counted in a gamma counter. Cytotoxicity was calculated as: percentage of cytotoxicity = 100 × ((cpm experimental release) − (cpm spontaneous release))/((total cpm) − (cpm spontaneous release)).

Conjugate formation. Triplicate mixtures of 2.5×10^5 effector cells and 2.5×10^5 target cells were centrifuged at 400 × *g* for 5 min and then incubated for 6 min at 37°C. The cells were resuspended in 0.2 ml of cold PBS by pipetting, and examined with a microscope. Effector cells and target cells were distinguished by size. Two hundred target cells were counted in each sample. Adhesion was expressed as the percentage of target cells found in conjugates.

Cell proliferation. Assays were performed in triplicate in flat bottomed microtiter plates. Cryopreserved CTL were thawed and plated at 2×10^4/well in RPMI 1640 supplemented with 15% human serum. A total of 1×10^5 2,000 rad-irradiated PBML or 1×10^4 10,000 rad-irradiated JY cells were then added to wells. Cells were incubated at 37°C for 48 h and then labeled with 0.5 μCi [^{3}H] thymidine per well. After additional incubation of 20 to 24 h cells were harvested and thymidine uptake was assessed by scintillation counting.

RESULTS

Our approach for selecting cell variants deficient in glycolipid anchored proteins was to generate DAF-negative cell lines and to identify DAF-negative clones, which also lack other PI-anchored cell surface proteins. DAF was elected for the primary selection of variants, because it is expressed on B lymphocytes in considerable amounts (19), and in contrast to LFA-3 exists solely in a PI-anchored form. JY B lymphoblastoid cells were mutagenized, and DAF-negative cells were selected by immunofluorescence cell sorting. Three sorts were performed, each one selecting for the lowest 0.5% of the cells stained with anti-DAF antibodies. The population obtained after the second sort consisted of 23% DAF-negative cells, whereas the third sort yielded a population of nearly 100% DAF-negative cells. Cloning of this population resulted in 97% DAF-negative clones and 3% DAF-positive clones. DAF-negative mutant clones were then analyzed for expression of BLAST-1 Ag. BLAST-1, a 45-kDa protein found on activated and transformed B lymphoblasts (20), was recently characterized as a PI-anchored protein (28). An immunofluorescence flow cytometry histogram of two representative clones (designated clone 5 and clone 33) is shown in Figure 1*A* and *B*. As a comparison the staining of a DAF-positive clone (clone 25), selected during the search for DAF-negative clones, and of the parent cell line JY, is shown (Fig. 1*C* and *D*). Clones 5 and 33 did not react with anti-DAF or with anti-BLAST-1, indicating that loss of surface DAF resulted from a defect in PI-anchorage, and thus was accompanied by deficiency of other PI-linked proteins. In contrast, mutant clones expressed normal levels of LFA-1, ICAM-1, HLA class I, and HLA class II molecules (data not shown).

LFA-3 is expressed on the cell surface of nucleated cells in both a polypeptide chain-anchored form and a PI-anchored form (13). To test the hypothesis that the mutant cells would express normal levels of the LFA-3 transmembrane form and be deficient in the LFA-3 PI-linked form, we studied LFA-3 biosynthesis in variant and wild-type lines. Cells were pulsed for 15 min with [^{35}S]methionine. One-half of each culture was chased with 1 mM unlabeled methionine for 1 h. Detergent lysates were

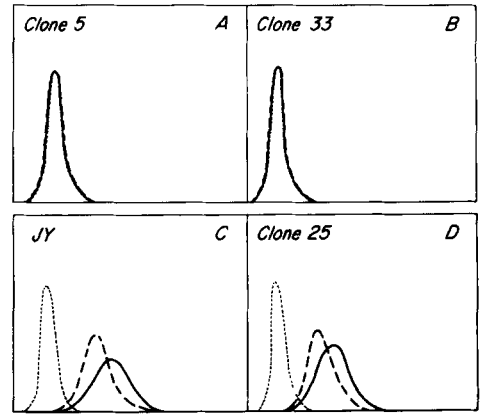

Figure 1. Fluorescent staining of DAF and BLAST-1 on wild-type and mutant cells. Cells were incubated with anti DAF mAb (*solid lines*), anti BLAST-1 mAb (*dashed lines*), or X63 control IgG (*dotted lines*), stained with fluorescein labeled goat anti-mouse IgG, and subjected to immunofluorescence flow cytometry.

then subjected to immunoprecipitation with anti-LFA-3-Sepharose followed by SDS-PAGE. A 15-min pulse of wild-type JY cells revealed two distinct LFA-3 precursors of 41 and 37 kDa (Fig. 2, *lanes 1* and *3*). The 41-kDa band has previously been found to correspond to the transmembrane LFA-3 form, whereas the 37-kDa band corresponds to the PI-linked LFA-3 form (13). After 1-h chase the precursors converted to the mature form of LFA-3 migrating as a broad band with mean size of 65 kDa (Fig. 2, *lanes 2* and *4*). *N*-glycanase treatment converted both the precursor and mature forms of LFA-3 to two bands of 29 kDa (p29) and 25.5 kDa (p25.5) (Fig. 2, *lanes 9* to *12*), corresponding to the polypeptide chain-attached and the PI-anchored LFA-3 species, respectively (13). Our results confirmed that the size heterogeneity of mature LFA-3 is due to *N*-linked carbohydrate, which causes the two forms of mature LFA-3 to overlap in SDS-PAGE (13).

The DAF-negative clones 5 and 33 revealed the 41-kDa precursor band, corresponding to the transmembrane LFA-3 form, seen in wild-type lines. However, the DAF-negative clones lacked the 37-kDa precursor band, corresponding to the PI-attached LFA-3 (Fig. 2, *lanes 5* and *7*). Instead, the mutant cells revealed two faint bands of 39 kDa and 36 kDa. The upper band may represent molecules that retain the C-terminal hydrophobic domain, whereas the lower band may consist of precursors from which this domain has been removed. These species were not detected in pulse-labeled wild-type cells, consistent with removal of the hydrophobic tail and attachment of the glycophospholipid structure being rapid post-translational processes (2, 3). After chase the mutant cells contained mature LFA-3 migrating as a broad band with mean size of 66 kDa (Fig. 2, *lanes 6* and *8*). Additionally, the mutant cells contained three LFA-3 bands at 42 kDa, 38 kDa, and 34 kDa not present in wild-type cells. These bands thus appear to be derived from the LFA-3 precursor that would normally be PI-anchored, and the smaller two forms may correspond to the 39-kDa and 36-kDa forms from which mannose has been trimmed during long residence in the rough endoplasmic reticulum (29).

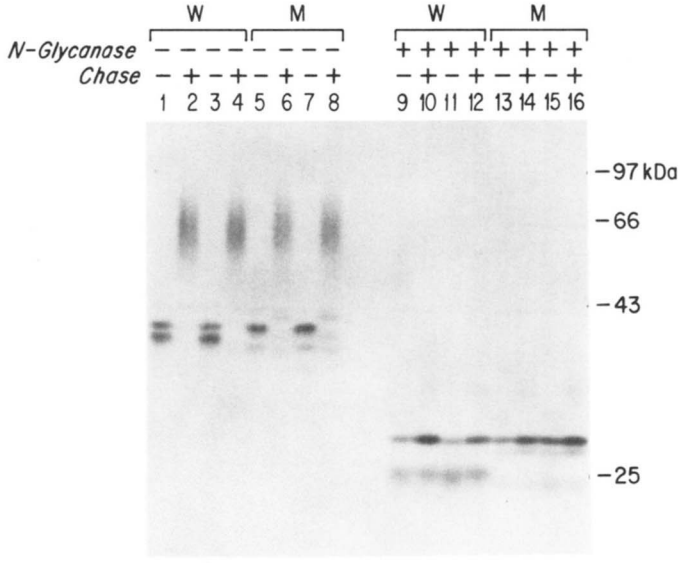

Figure 2. Biosynthesis of LFA-3. Wild-type (*W*) (clones 2 and 25) and mutant (*M*) (clones 5 and 33) JY cells were labeled with ^{35}S with or without a 1-h chase. LFA-3 was immunoprecipitated from cell lysates with anti-LFA-3-Sepharose and incubated with or without *N*-glycanase as described in *Materials and Methods*. LFA-3 samples were subjected to reducing 10% SDS-PAGE and fluorography.

N-glycanase digestion of both the precursor and the mature form of LFA-3 in mutant cells gave rise to p29, corresponding to the transmembrane species. However, p25.5, corresponding to the PI-attached form, was missing (Fig. 2, *lanes 13* to *26*), being replaced by faint bands of 27.5 kDa and 24.5 kDa. These two bands may correspond to PI-anchored LFA-3 intermediates (or aberrant proteins) containing (p27.5) or lacking (p24.5) the hydrophobic peptide tail.

We further studied LFA-3 expression on the cell membrane. As shown in Table I, levels of LFA-3 in mutant cells were about 50% lower than in control cells. Furthermore, although treatment with PIPLC released 39% of LFA-3 from control cells, no LFA-3 was released from PIPLC-treated mutant cells (Table 1), suggesting that the PIPLC-sensitive PI-linked form of LFA-3 was not expressed by the mutant lines. This was confirmed by PIPLC treatment of surface-iodinated cells. LFA-3 released by PIPLC was isolated from the medium and LFA-3 remaining on the cell surface was isolated from cell detergent lysates. A portion of cell-surface LFA-3 was released from control cells by PIPLC (Fig. 3, *lane 13*). However, no LFA-3 was released from mutant cells by PIPLC (Fig. 3, *lane 15*). To resolve which form was released, isolated LFA-3 was digested with *N*-glycanase. The p29 band was more intense than the p25.5 band when surface iodination was used to label JY cells, and remained on the cell surface after PIPLC treatment of control or mutant cells (Fig. 3, *lanes 2, 4, 6,* and *8*). From control cells PIPLC released p25.5 and no p29 (Fig. 3, *lane 14*), as previously described (13). In contrast, p25.5 was not released from mutant cells (Fig. 3, *lane 16*). The amount of released material was greater than expected based on the amount of cellular p25.5. The greater recovery of the PI-anchored form after removal of PI may be caused by better solubilization or more efficient binding to mAb, and has been previously observed (13).

To confirm the absence of the PI-anchor, cells were labeled with [^{3}H]palmitic acid or with [^{3}H]ethanolamine, and LFA-3 was immunoprecipitated from cell lysates (Fig. 4). We consistently observed that upon short exposures of autoradiograms only LFA-3 of wild-type cells seemed to be labeled with [^{3}H]palmitate, whereas longer exposures revealed much lower incorporation of palmitate into LFA-3 of mutant cells as compared to wild-type

TABLE I
LFA-3 expression after PIPLC treatment[a]

Cells	Treatment	LFA-3 (SLFI)[b]	Decrease of Surface LFA-3 (%)
Clone 25 (DAF-positive)	None	15.5	
	PIPLC	9.4	39
Clone 5 (DAF-negative)	None	8.1	
	PIPLC	7.9	3
Clone 33 (DAF-negative)	None	7.3	
	PIPLC	7.3	0

[a] Cells were treated with 10 μg/ml of PIPLC as described in *Materials and Methods*. The cells were washed and stained with LFA-3 mAb and fluorescein-labeled goat anti-mouse IgG. Stained cells were analyzed by immunofluorescence flow cytometry.

[b] The specific linear fluorescence intensity (SLFI) of cells treated under the same conditions but without PIPLC was taken as 100%.

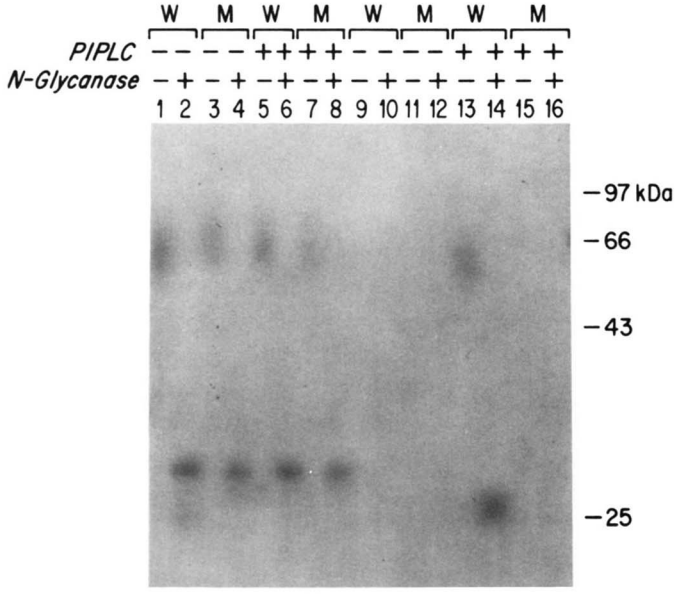

Figure 3. SDS-PAGE of LFA-3 released from cells by PIPLC. Surface-iodinated clone 25 wild-type cells (*W*) and clone 5 mutant cells (*M*) were incubated with or without PIPLC (10 g/ml) for 1 h at 37°C. Supernatants were centrifuged at 100,000 × *g* for 1 h. LFA-3 was immunoprecipitated from cell lysates (*lanes 1* to *8*) and from the supernatants (*lanes 9* to *16*) and treated with or without *N*-glycanase. LFA-3 samples were subjected to reducing 10% SDS-PAGE and autoradiography.

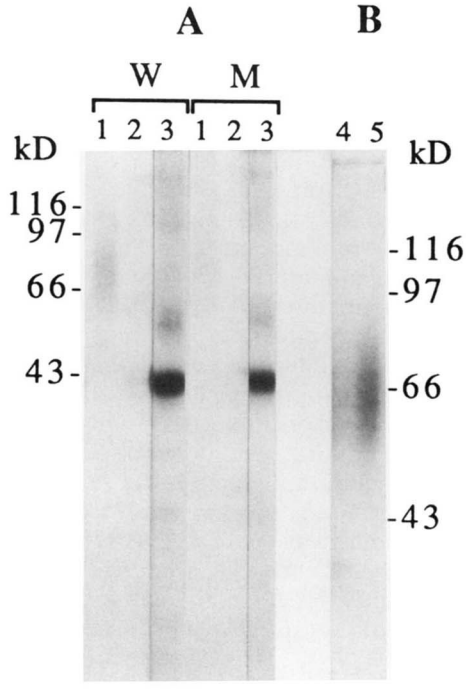

Figure 4. Ethanolamine and palmitic acid incorporation into LFA-3. Wild-type (*W*) and mutant (*M*) JY cells were labeled for 16 h with [^{3}H] palmitic acid (*lanes 1* to *3*), or for 24 h with [^{3}H]ethanolamine (mutant, *lane 4*; wild type, *lane 5*). LFA-3 (*lanes 1*, *4*, and *5*), LFA-1 (lanes *2*), and HLA (*lanes 3*) were immunoprecipitated from cell lysates with mAb-Sepharose and subjected to nonreducing 10% SDS-PAGE. Much or all of the apparent exposure in *lane 4* is caused by much more radioactive [^{35}S] methionine-labeled LFA-3 in an adjacent lane.

cells (Fig. 4*A*, *lanes 1*). Under the same conditions palmitate labeling of HLA from wild-type and mutant cells was comparable (Fig. 4, *lanes 3*). Fatty acylation appears to be a common post-translational modification of membrane proteins (30–32). It has been reported (31) that palmitic acid is covalently linked via a thioester bond to transmembrane cysteines of HLA in JY cells. Labeling of HLA with palmitic acid was reproduced in our study. The presence of a cysteine in the transmembrane hydrophobic region of the polypeptide chain-anchored form of LFA-3 (15), which is fatty acylated, may explain the weak labeling of mutant cells. The PI-anchor is attached to the polypeptide COOH-terminus through ethanolamine, which thus constitutes the proximal end of the anchor. Labeling experiments with [^{3}H]ethanolamine showed that wild-type LFA-3 incorporated ethanolamine, whereas mutant cell LFA-3 incorporated little or no ethanolamine (Fig. 4*B*, *lanes 4* and *5*), indicating that LFA-3 of mutant cells lack the PI-anchor.

The present results indicate that mutant cells synthesize LFA-3, but do not express its PI-anchored form, probably as a result of their inability to synthesize a critical component of the anchor or to attach the anchor to the polypeptide. The fate of the unexpressed LFA-3 was therefore examined. Cell lysates and culture media from cells labeled for 6 h with [^{35}S]methionine were immunoprecipitated with anti-LFA-3-Sepharose or with anti-HLA-Sepharose and subjected to SDS-PAGE. Cellular and extracellular samples derived from equal numbers of cells were compared (Fig. 5). LFA-3 was secreted into the medium by both wild-type and mutant cells; however, the mutant cells secreted much more LFA-3 than wild-type cells (Fig. 5*A*, *lanes 5* to *8*). Secreted LFA-3 in both wild-type and mutant cells revealed a mean apparent m.w. of 50 kDa, which is lower than the 65-kDa cell-surface LFA-3. *N*-glycanase converted the secreted LFA-3 to p24.5 (Fig. 5*A*, *lanes 13* to *16*), suggesting that the lower m.w. of secreted LFA-3 was caused by altered glycosylation. The p24.5 backbone of secreted LFA-3 migrated identically to p24.5 in mutant cells (Fig. 5*A*, *lanes 11* to *16*), suggesting that these protein backbones may be identical. Of the two aberrant forms of LFA-3 found in mutant cells, p24.5 and p27.5 (Fig. 5*A*, *lanes 11* and *12*, and Fig. 2), only p24.5 was secreted, consistent with the idea that p24.5 may be related to p27.5 by removal of the hydrophobic C-terminal peptide. Membrane shedding could not account for accumulation of LFA-3 in culture media because: a) cell supernatants were centrifuged before immunoprecipitation for 1 h at 100,000 × *g*; b) the polypeptide chain-anchored form of LFA-3 was not detected in the medium; c) HLA class I was not detected in culture supernatants (Fig. 5*B*). It therefore appears that removal of the C-terminal hydrophobic polypeptide chain predicted by the cDNA sequence, together with failure to attach the glycosyl-PI anchor results in secretion of a p24.5 form of LFA-3. The same p24.5 is secreted in small amounts by wild-type cells.

Recent studies demonstrated that LFA-3 functions as a ligand for CD2 (7, 8) and that CD2-LFA-3 interaction mediates CTL-target cell adhesion (6). Predicted high diffusion rate of PI-linked LFA-3 might render it more effective than the transmembrane form in mediating cell adhesion (22). To address the question of whether the PI-anchored LFA-3 plays an essential role in adhesion we analyzed the capacity of JY mutant cells to interact with CTL. The CTL clone 8.2 is specific for HLA-DPw2, which is expressed on JY cells. As shown in Figures 6 and 7, both wild type (clone 25) and mutant (clone 5) JY cells formed conjugates with the effector cells, and both were subsequently lysed to a similar extent. Conjugate forma-

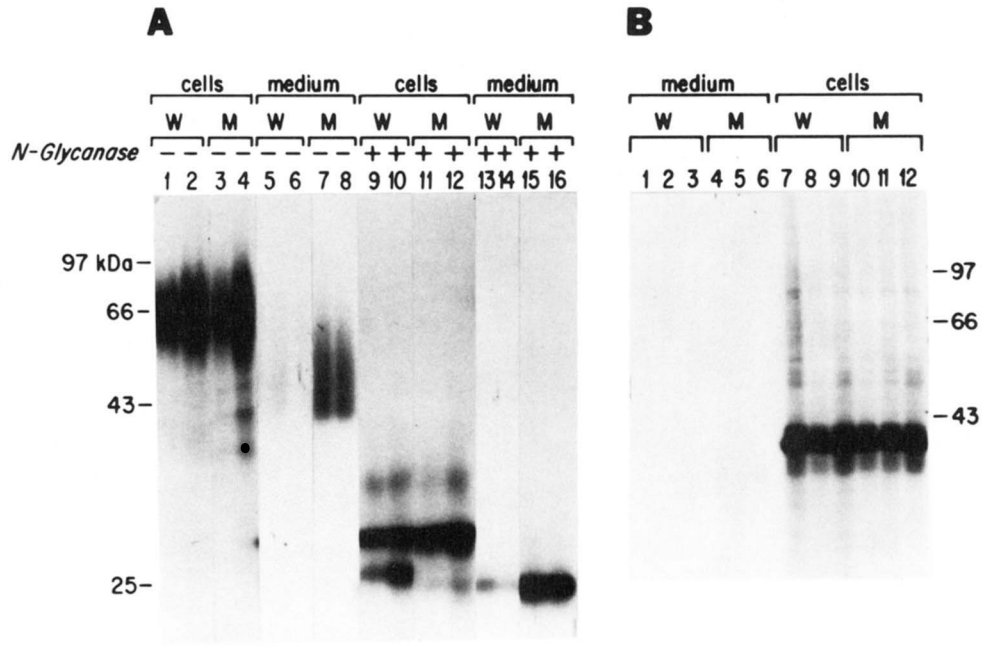

Figure 5. Secretion of LFA-3 by wild-type (*W*) and mutant (*M*) JY cells. Two DAF-positive wild-type clones (2 and 25) and two DAF-negative mutant clones (5 and 33) were biosynthetically labeled with [^{35}S]methionine for 6 h. Culture supernatants were centrifuged at 100,000 × *g* for 1 h. LFA-3 and HLA were immunoprecipitated from cell lysates and from culture media. Immunoprecipitated LFA-3 was treated with or without *N*-glycanase. LFA-3 samples (Fig. 5*A*) and HLA samples (Fig. 5*B*) were then subjected to reducing 10% SDS-PAGE and fluorography.

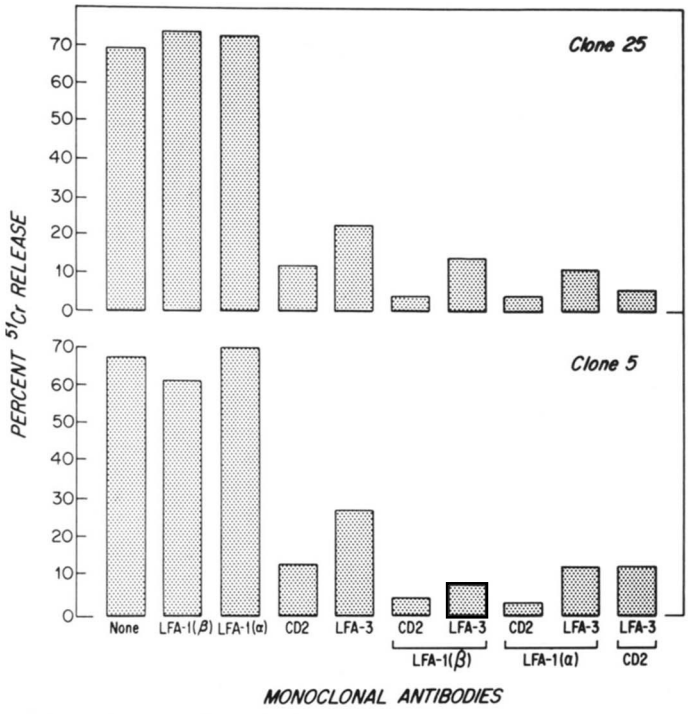

Figure 6. Cytolysis of wild-type and mutant JY cells and its inhibition by mAb. Ag-specific CTL 8.2 cells were incubated for 4 h with [^{51}Cr]-labeled wild-type (clone 25) or mutant (clone 5) JY cells, at E:T ratio of 5:1. mAb (ascites fluids) were present during the assay at final concentration of 1:200. The anti-LFA-1 β-subunit antibody was TS1/18, the anti-LFA-1 α-subunit antibody was TS1/22, the anti-CD2 antibody was TS2/18, and the anti-LFA-3 antibody was TS2/9.

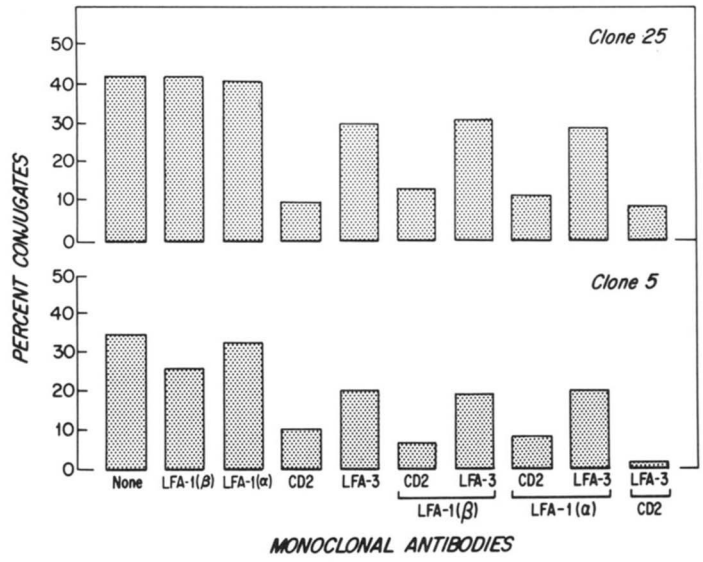

Figure 7. Conjugate formation of CTL with wild-type and mutant JY cells and its inhibition by mAb. CTL 8.2 cells were mixed at 1:1 ratio with wild-type (clone 25) or mutant (clone 5) cells. Conjugate formation was assessed after pelleting, incubation for 6 min at 37°C, and resuspension in cold PBS. mAb (see Fig. 6) were added at final concentration of 1/200.

tion by variant cells was slightly lower than that observed for wild-type cells. This difference may be attributed to the overall level of LFA-3 expression, which was 40 to 50% lower in mutant JY (Table I). Correlation between levels of LFA-3 surface expression on target cells and extent of conjugate formation has been reported (33). Blocking studies with mAb demonstrated marked inhibition of both conjugate formation and cytolysis by anti-CD2 and anti-LFA-3 mAb, but only a marginal effect of anti LFA-1 mAb (Figs. 6 and 7), indicating that the CD2-LFA-3 pathway is predominant in interaction of JY cells with CTL. Wild-type and mutant JY cells were similarly affected by the blocking CD2 and LFA-3 mAb, suggesting that the transmembrane form of LFA-3 can bind effectively to CD2 and mediate potent adhesion in the absence of its PI-anchored counterpart.

In addition to its important role in adhesion and cytolysis, CD2-LFA-3 interaction is also involved in T cell activation (34, 35). We therefore studied whether the absence of PI-linked LFA-3 affects stimulation of the CTL 8.2 clone by JY cells. No significant difference was observed in the capacity of wild-type and mutant JY clones to stimulate CTL proliferation (Table II). Similar results were obtained with lower numbers of stimulating cells (data not shown), indicating that the transmembrane

TABLE II

Wild-type and mutant JY clones trigger CTL proliferation[a]

Cells	CPM
CTL	680 ± 73
CTL + DPw2⁻ PBML	1,770 ± 115
CTL + DPw2⁺ PBML	65,384 ± 3,002
CTL + JY clone 25 (wild type)	50,229 ± 4,678
CTL + JY clone 5 (mutant)	48,375 ± 4,090
DPw2⁻ PBML	391 ± 45
DPw2⁺ PBML	442 ± 59
JY clone 25	972 ± 71
JY clone 5	1,223 ± 66

[a] 2×10^4 CTL/well were incubated with 1×10^5 2,000 rad-irradiated PBML or 1×10^4 10,000 rad-irradiated JY cells. [^{3}H]Thymidine (0.5 μCi) was added at 48 h, and cells were harvested after additional incubation for 20 h.

LFA-3 is able to function in activation in the absence of the PI-anchored form.

DISCUSSION

We have selected human B lymphoblastoid mutant cells, which are deficient in DAF, BLAST-1, and the PI-anchored form of LFA-3. The deficiency in three PI-attached proteins suggests that the primary defect of these mutant cells is related to the assembly or the linkage of the PI glycolipid moiety. Because both the proximal (ethanolamine) and the distal (fatty acid) ends of the anchor are deficient in mutant LFA-3, it is most probable that the entire anchor is absent.

A similar generalized defect in expression of PI-anchored proteins occurs in an acquired disease, PNH. PNH is one of few known diseases that involve an acquired error of metabolism. The defect is clonal and does not appear to be caused by an infectious agent because it can be cured by bone marrow transplantation (36). E from PNH patients are unusually susceptible to autologous C as a result of the absence of two PI-anchored proteins that regulate C assembly on cell surfaces, DAF (37, 38), and homologous restriction factor (39). The mutant cell lines described in the present paper may provide an experimental model for studying the PNH phenotype in nucleated cells. Thus, the sensitivity of JY mutant cells to autologous C is 10-fold higher than that of wild-type JY cells, which are relatively resistant to human C (manuscript in preparation). It is possible, however, that in spite of their identical phenotype, PNH patient cells and JY mutant cells differ in their genetic defect. Because the anchor biosynthesis and attachment are complex processes and involve several types of carbohydrate linkages not found in other eukaryotic carbohydrates (40), a large number of enzymes may be potential mutagenic targets. Thus, it is possible that distinct JY mutant clones, as well as distinct PNH patients, differ in their specific biosynthetic block. Complexity in glycosyl-PI biosynthesis is suggested by the series of murine mutant lymphoma cell lines that are deficient in Thy-1 expression (41–47). These were classified into eight complementations classes of which only class D is deficient in the Thy-1 structural gene. In all the others the Thy-1 protein is synthesized but not expressed on the cell surface, and at least some of these seven mutant classes have defects in anchor synthesis or addition.

Our results confirm previous observations that LFA-3 exists in nucleated cells in two alternative forms (13) and that the large form (p29) is a transmembrane protein, whereas the smaller form (p25.5) is anchored to the plasma membrane by PI-linkage. Complementary DNA clones corresponding to both forms of the molecules have been obtained (14, 15). The deduced LFA-3 sequences for the PI-linked form and the polypeptide-linked form are identical from the amino terminus to the end of the hydrophobic membrane-spanning domain, differing only in two amino acids of the hydrophobic transmembrane domain and in the absence or the presence of a cytoplasmic domain. The ability of PIPLC to release the PI-anchored form of LFA-3 shows that the C-terminal hydrophobic sequence predicted by the cDNA clone is not present in the mature protein (13, 14) as has been demonstrated for other PI-anchored proteins (2, 3). Studies on representative PI-anchored proteins have shown that ethanolamine of the PI glycan moiety is linked via an amide bond to the C-terminal amino acid of the mature protein, suggesting that removal of the hydrophobic C-terminal precursor peptide is tightly linked to addition of the PI glycan anchor (1–3, 40, 48).

At least two forms of LFA-3 were detected in mutant cells that correspond either to aberrant or normal intermediates in the biosynthesis of the PI anchored form. These precursors of 39 and 36 kDa were reduced to 27.5 kDa (p27.5) and 24.5 kDa (p24.5) by *N*-glycanase. The precursor corresponding to p24.5 (probably the one of 36 kDa) could be secreted, and was converted to a mature size of 50 kDa. Its size in SDS-PAGE suggests considerably less carbohydrate addition during processing than for mature cell surface LFA-3 (65 kDa). This may reflect faster transit through the Golgi, different accessibility to membrane-bound enzymes, or a different transit route for a soluble as opposed to a membrane-bound glycoprotein. Identical results were obtained for small amounts of LFA-3 secreted by wild-type cells.

Based on the size difference, the secretion of p24.5 but not p27.5, and deficient incorporation of palmitate and ethanolamine into LFA-3 in mutant cells, it is tempting to speculate that p27.5 retains the C-terminal hydrophobic peptide, whereas p24.5 lacks this peptide. An educated guess as to the location of this cleavage site (48) predicts loss of 3.2 kDa, in excellent agreement with the difference in size between p27.5 and p24.5. If the p27.5 is indeed the uncleaved LFA-3 precursor, it should as well be present in wild-type cells. However, it may not be detected in normal cells, because cleavage of the hydrophobic domain and attachment of the glycophospholipid structure are very rapid post-translational processes (2, 3). The cleavage process may be slowed down in the mutant cells because of lack of the glycolipid moiety. Further studies are required to characterize the LFA-3 intermediates in the mutant cells.

Although the defects of the PI-anchor-deficient JY cell lines are yet unknown, a comparison between the presently studied JY mutants (clones 5 and 33) and the murine Thy-1 mutants may be outlined. Class E Thy-1 mutant lines lack synthesis of dolichol-P-mannose (42), suggesting that dolichol-P-mannose is the sugar donor of at least one step in the anchor synthesis. Because the JY mutant cells are not deficient in conversion of guanosine 5′-diphosphate-mannose to dolichol-P-mannose (data not shown), they differ in their lesion from class E Thy-1 mutants. In class A, C, F, and H Thy-1 mutants the intracellular Thy-1 retains the hydrophobic C-terminal peptide, which is not replaced by a glycolipid anchor (46,

47). These mutant lines do not secrete Thy-1. In contrast, class B mutants produce hydrophilic Thy-1 molecules, which are 2 kDa smaller than the Thy-1 of the other cell lines, are rapidly secreted (47), and are very weakly labeled by ethanolamine (46).

The physiologic significance of the PI-anchor is yet unknown. A minimal hypothesis for the function of PI tails is membrane attachment per se. However, the fact that LFA-3 and NCAM are expressed in both transmembrane and PI-anchored forms (4, 13) suggests that expression of adhesion molecules with different types of membrane anchorage is of functional significance. The presently described mutant cells, which express only one form of LFA-3, may elucidate the division of LFA-3 function between the two forms. In the present paper we have demonstrated that wild-type and mutant cells interact with CD2 to similar extents, indicating that the transmembrane form can mediate adhesion and is functionally competent to support T cell-mediated killing and T cell activation. Molecules with polypeptide anchors often show very slow diffusion rates because of interactions with other membrane molecules or cytoskeletal components (49). In contrast, several PI-anchored proteins show relatively high diffusion coefficients (50, 51). Therefore, it could be hypothesized that a predicted high diffusion rate of PI-linked LFA-3 may facilitate diffusion to sites of contact with its ligand CD2 and thus may promote cell-cell interactions, whereas the transmembrane form of LFA-3 may be involved in another function, such as signal transduction. However, the present data indicate that the polypeptide transmembrane form of LFA-3 is fully capable of mediating cell adhesion interactions. Because human E LFA-3 is found only in the PI-anchored form (13, 22) and can mediate cell adhesion (8), it seems that the two distinct membrane-anchored forms of LFA-3 can bind CD2 and mediate adhesion. In addition to triggering other cells via CD2, LFA-3 may also transduce signals to the cells that display it, because binding of anti-LFA-3 antibodies induces IL-1 release by monocytes and thymic epithelial cells (16). The possibility that one of the LFA-3 forms is involved in signal reception or transduction, or that ligand-mediated co-clustering of the two forms leads to signaling, is now under investigation.

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